

Disruption of Astral Microtubule Contact with the Cell Cortex Activates a Bub1, Bub3, and Mad3-dependent Checkpoint in Fission Yeast[□] [▽]

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In animal and yeast cells, the mitotic spindle is aligned perpendicularly to the axis of cell division. This ensures that sister chromatids are separated to opposite sides of the cytokinetic actomyosin ring. In fission yeast, spindle rotation is dependent upon the interaction of astral microtubules with the cortical actin cytoskeleton. In this article, we show that addition of Latrunculin A, which prevents spindle rotation, delays the separation of sister chromatids and anaphase promoting complex-mediated destruction of spindle-associated Securin and Cyclin B. Moreover, we find that whereas sister kinetochore pairs normally congress to the spindle midzone before anaphase onset, this congression is disrupted when astral microtubule contact with the actin cytoskeleton is disturbed. By analyzing the timing of kinetochore separation, we find that this anaphase delay requires the Bub3, Mad3, and Bub1 but not the Mad1 or Mad2 spindle assembly checkpoint proteins. In agreement with this, we find that Bub1 remains associated with kinetochores when spindles are mispositioned. These data indicate that, in fission yeast, astral microtubule contact with the medial cell cortex is monitored by a subset of spindle assembly checkpoint proteins. We propose that this checkpoint ensures spindles are properly oriented before anaphase takes place.

INTRODUCTION

In eukaryotes, the separation of sister chromatids is mediated by the interaction of spindle microtubules with specialized regions of chromosomes known as kinetochores. At the start of prometaphase, kinetochores are not attached to microtubules. The kinetochore of one sister chromatid then captures a microtubule nucleated from a spindle pole. Once its sister kinetochore has captured microtubules from the other pole, the chromosome becomes bioriented. During metaphase, all bioriented chromosomes move to the equatorial plane, known in animal cells as the metaphase plate, in a process called chromosome congression (Rieder and Salmon, 1994). At anaphase, cohesion is lost, allowing sister chromatids to separate to their respective poles. Finally, the cytokinetic actomyosin ring contracts perpendicularly to, and at the site of, the spindle midzone to ensure that each set of sister chromatids is separated to daughter cells.

Cell cycle progression in all eukaryotic cells is monitored by a series of checkpoints that ensure both the fidelity and the temporal and spatial order of cell cycle events (Hartwell and Weinert, 1989). One of the best studied of these is a checkpoint that monitors the assembly of the mitotic spindle (Yu, 2002; Zhou *et al.*, 2002; Cleveland *et al.*, 2003). Components of this checkpoint, which is often referred to as the spindle assembly checkpoint (SAC), were first identified in budding yeast and include the Mad1, Mad2, Mad3, Bub1, Bub3, and Mps1 proteins (Li and Murray, 1991; Hoyt *et al.*, 1991; Weiss and Winey, 1996). Structural and functional homologues of these proteins have been identified in all other eukaryotes so far examined, including fission yeast (He *et al.*, 1997, 1998; Bernard *et al.*, 1998; Ikui *et al.*, 2002; Millband and Hardwick, 2002). In response to microtubule-disrupting agents, these proteins translocate to kinetochores and inhibit the anaphase promoting complex (APC) (Chen *et al.*, 1996; Li and Benezra, 1996), an E3 ubiquitin ligase that is responsible for the destruction not only of Cyclin B but Securin, an inhibitor of Separase (Funabiki *et al.*, 1996b; Zachariae and Nasmyth, 1999). Separase cleaves Scc1/Rad21, a component of the Cohesin complex that holds sister chromatids together (Uhlmann *et al.*, 1999; Tomonaga *et al.*, 2000; Uhlmann *et al.*, 2000). Microtubule-disrupting agents thus block anaphase onset by inhibiting the activation of Separase.

The molecular nature of the defect that is sensed at kinetochores is not clear. Two models have been put forward. In the first (attachment model), anaphase is initiated when all kinetochores are bound to a bipolar spindle (Rieder *et al.*, 1994, 1995). In the second (tension model), anaphase is initiated only when balanced tension is exerted upon kinetochores when chromosomes congress to the metaphase plate

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(McIntosh, 1991, Li and Nicklas, 1995; Stern and Murray, 2001). Originally, it was thought that Mad2 was the key effector of the SAC, because Mad2 interacts with and inhibits Cdc20 (Fizzy/Slp1), an activator of the APC (Hwang *et al.*, 1998; Kim *et al.*, 1998). However, the situation now seems more complex. Although heterodimeric complexes of Mad1-Mad2, BubR1(Mad3)-Bub3, and Bub1-Bub3 exist in interphase cells, higher order complexes are formed in mitosis and in response to microtubule-disrupting agents (Chen *et al.*, 1999; Hardwick *et al.*, 2000; Millband and Hardwick, 2002). In particular, a mitotic checkpoint complex has been purified from human cells that contains the Mad2, Cdc20, BubR1(Mad3), and Bub3 proteins and is a significantly more potent inhibitor of APC in vitro than Mad2 alone (Sudakin *et al.*, 2001; Fang, 2002). A similar complex, containing Mad2, Cdc20, Mad3, and Bub3, has been identified in yeast (Hardwick *et al.*, 2000; Millband and Hardwick, 2002). Independently, Tang *et al.* (2001) have isolated a distinct inhibitory complex that contains Cdc20, BubR1, and Bub3 but not Mad2. Intriguingly some spindle assembly checkpoint proteins, including Bub1, BubR1(Mad3), and Bub3 are present at kinetochores as chromosomes congress at the metaphase plate, whereas Mad1 and Mad2 are not (Waters *et al.*, 1998; Hoffman *et al.*, 2001). One possibility is that a distinct set of SAC components monitor bipolar attachment of kinetochores to spindle poles and chromosome congression.

We previously proposed that, in fission yeast, a spindle orientation checkpoint (SOC) delays anaphase onset when astral microtubule interaction with the medial cell cortex and thereby spindle rotation is disturbed (Gachet *et al.*, 2001, 2004). Recently, Rajagopalan *et al.* (2004) have suggested, by analysis of a mutant in the kendrin-like protein Pcp1, that metaphase spindle alignment is monitored at the spindle pole by the Bub1 and Mph1 but not Mad1, Mad2, Mad3, or Bub3 checkpoint proteins. This is curious because first, both Mad3 and Bub3 are necessary for Mps1(Mph1) to impose a metaphase arrest in budding yeast (Hardwick *et al.*, 1996), and second, Bub3 is required both for the association of Bub1 to kinetochores and for the checkpoint function of Bub1 in yeast and mammalian cells (Farr and Hoyt, 1988; Taylor *et al.*, 1988; Warren *et al.*, 2002; Gillett *et al.*, 2004). In this article, we have reassessed the mechanism by which spindle position is monitored in fission yeast by analyzing kinetochore dynamics when contact of astral microtubules with the medial cell cortex is disturbed.

MATERIALS AND METHODS

Cell Culture

Media, growth, and maintenance of strains were as described previously (Moreno *et al.*, 1991). Strains used in this study are listed in Table 1. Cell cultures were grown at 28°C in YES unless otherwise stated. Latrunculin A was purchased from Molecular Probes (Eugene, OR), dissolved in dimethyl sulfoxide at a stock concentration of 1 mg/ml. All strains were *leu1-32 ura4-D18* unless otherwise stated. *ade6-* is either *ade6-M210* or *ade6-M216*. Cultures of *nmt1-gfp-atb2* cells were grown at 28°C in minimal medium, and thiamine was added at a concentration of 3.75g/l to partially repress expression of *nmt1-gfp-atb2*. In these conditions, *nmt1-gfp-atb2* cells grew at a rate indistinguishable from wild type.

Epitope Tagging

Carboxy-terminal green fluorescent protein (GFP) and cyan fluorescent protein (CFP) epitope tagging of Cdc11p and Ndc80p were done by polymerase chain reaction-based gene targeting (Bähler *et al.*, 1998). The growth rates of *cdc11-CFP*, *ndc80-GFP*, and *cdc11-CFP ndc80-GFP* cells were indistinguishable from wild type. Compound tagged and mutant strains were constructed by standard genetic methods (Moreno *et al.*, 1991).

Cell Synchronization

Cell synchrony was achieved either by centrifugal elutriation or lactose gradient size selection. Cells were resuspended in fresh medium at 10⁶ cells/ml

Table 1. Strains used in this study

Strain no.	Genotype	Source
JM2418	<i>h⁺ plo1-GFP:kanR</i>	D. McCollum
YG 309	<i>h⁻ GFP-nmt1-atb2(lys1)</i>	D-Q. Ding
JM2591	<i>h⁻ ndc80-GFP:kanR</i>	J. Kilmartin
JM2590	<i>h⁺ cdc13-GFP(LEU2)</i>	M. Yanagida
JM2564	<i>h⁺ cut2-364:cut2-GFP(LEU2)ura4⁺</i>	M. Yanagida
JM2379	<i>h⁺ mad1::ura4</i>	T. Matsumoto
AE148	<i>h⁻ mad2::ura4</i>	T. Matsumoto
JM2324	<i>h⁻ mad3::ura4</i>	K. Hardwick
JM2323	<i>h⁻ bub1::ura4</i>	K. Hardwick
JM2325	<i>h⁻ bub3::ura4</i>	K. Hardwick
SS560	<i>h⁻ mph1::ura4 ade6-</i>	S. Sazer
JM2747	<i>h⁺ bub1(K762M) lys1-1 his7-366 ade6-</i>	J.-P. Javerzat
JM2748	<i>h⁺ mad2-GFP(LEU2) ura4-D18</i>	T. Toda
MA239	<i>h⁺ bub1-GFP:kanR</i>	T. Toda
JM2608	<i>h⁺ cdc11-CFP:kanR</i>	This study
JM2746	<i>h⁺ cut2-GFP(LEU2) cdc11-CFP:kanR</i>	This study
JM2763	<i>h⁻ ndc80-GFP:kanR cdc11-CFP:kanR</i>	This study
JM2751	<i>h⁻ mad2::ura4 cdc13-GFP(LEU2)</i>	This study
JM2752	<i>h⁺ bub1::ura4 cdc13-GFP(LEU2) ade6-</i>	This study
JM2891	<i>h⁻ mad1::ura4 ndc80-GFP:kanR cdc11-CFP::kanR</i>	This study
JM2749	<i>h⁺ mad2::ura4 ndc80-GFP:kanR cdc11-CFP:kanR</i>	This study
JM2892	<i>h⁻ mad3::ura4 ndc80-GFP:kanR cdc11-CFP::kanR</i>	This study
JM2750	<i>h⁺ bub1::ura4 ndc80-GFP:kanR cdc11-CFP:kanR</i>	This study
JM2889	<i>h⁻ bub3::ura4 ndc80-GFP:kanR cdc11-CFP:kanR</i>	This study
JM2890	<i>h⁺ mph1::ura4 ndc80-GFP:kanR cdc11-CFP::kanR</i>	This study
JM2896	<i>h⁻ bub1(K762M) ndc80-GFP:kanR cdc11-CFP::kanR</i>	This study
JM2757	<i>h⁻ mad2-GFP(LEU2) ndc80-CFP:kanR</i>	This study
JM2758	<i>h⁻ mad2-GFP(LEU2) cdc11-CFP:kanR</i>	This study
JM2759	<i>h⁻ bub1-GFP:kanR ndc80-CFP:kanR</i>	This study
JM2760	<i>h⁺ bub1-GFP:kanR cdc11-CFP:kanR ade6-</i>	This study
JM2761	<i>h⁺ cps8-188 ndc80-GFP:kanR</i>	This study
JM2762	<i>h⁻ cdc11-123 ndc80-GFP:kanR ade6-</i>	This study
JM2808	<i>h⁻ bub1::ura4 cps8-188 ndc80-GFP:kanR</i>	This study
JM2809	<i>h⁻ bub1::ura4 cdc11-123 ndc80-GFP:kanR</i>	This study

All strains are *leu1-32 ura4-D18* unless otherwise stated. *ade6-* is either *ade6-M210* or *ade6-M216*.

and released at 28°C unless otherwise stated. The peak synchrony of septation was >35% in each experiment in the absence of drug.

Cell Fixation

plo1-GFP, *ndc80-GFP*, *ndc80-CFP*, *cdc11-CFP*, *mad2-GFP*, and *bub1-GFP* cells were fixed in 3.7% formaldehyde for 10 min at room temperature. *cdc13-GFP* cells were fixed in 100% cold methanol for 8 min and washed in phosphate-buffered saline. *cut2-GFP* cells were observed by live imaging. Actin rhodamine-phalloidin staining and tubulin immunofluorescence were performed as described previously (Marks and Hyams, 1985).

Microscopy

Live analysis of cells was performed in an imaging chamber (CoverWell PCI-2.5; Grace Bio-Labs, Bend, OR) filled with 1 ml of 1% agarose in minimal medium with or without 1.25 μM Latrunculin A and sealed with a 22 × 22-mm glass coverslip. Fluorescence microscopy was performed on a DeltaVision Spectris system containing a photometrics CH350L liquid cooled charge-coupled device camera and Olympus IX70 inverted microscope with a 100× objective equipped with Deltavision data collection system (Applied Precision, Issaquah, WA). Stacks of six Z-sections (0.35 μm apart) were taken at each time point with exposure times of 1 s for both GFP and CFP. Projected images were made for each time point followed by intensity adjustments and conversion to 24 bit TIFF images. The position of the spindle poles and

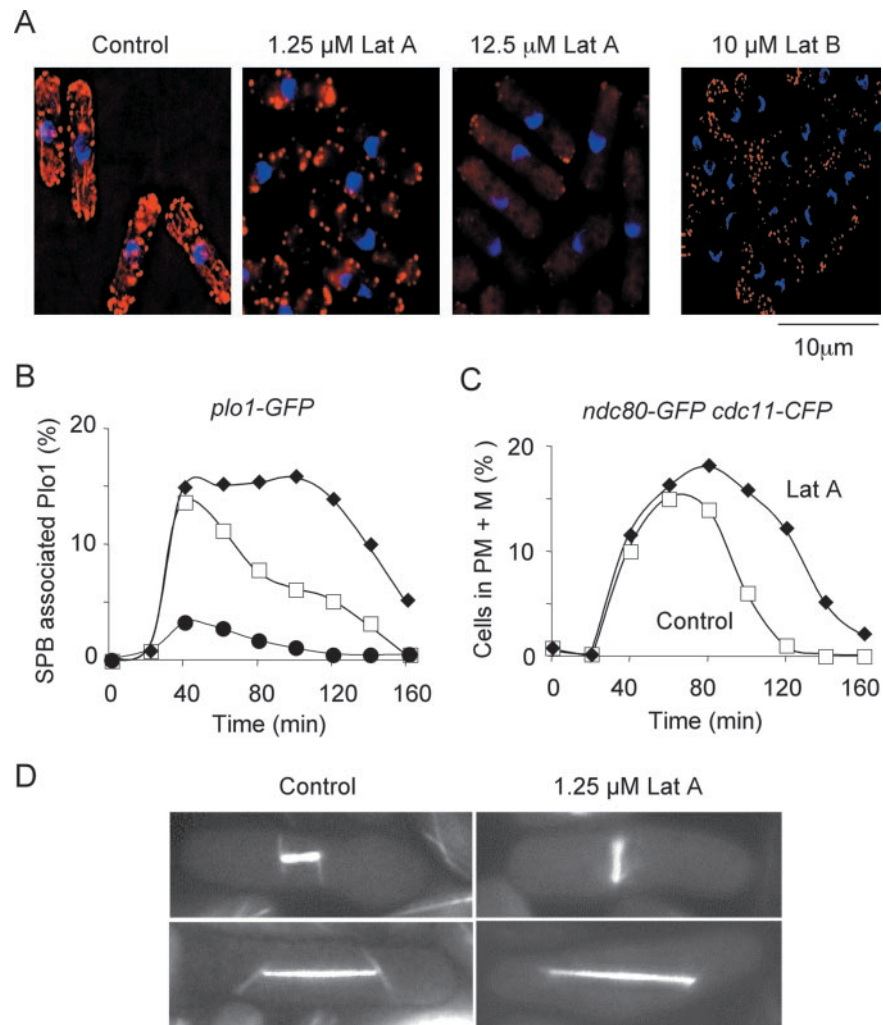


Figure 1. Selective disruption of actin cables delays the separation of sister chromatids without inhibiting mitotic entry. (A) Log phase cultures of wild-type cells were either untreated (control) or treated with 1.25 μ M Latrunculin A, 12.5 μ M Latrunculin A, or 10 μ M Latrunculin B for 15 min and stained for actin containing structures. (B) *plo1-gfp* cells were synchronized and incubated in the absence (open squares) or presence of either 1.25 μ M Latrunculin A (closed diamonds) or 12.5 μ M Latrunculin A (closed circles). The percentage of cells with Plo1-GFP on both SPBs was determined at the times shown ($n = 150$). (C) Synchronized *ndc80-gfp cdc11-cfp* cells were incubated in the absence (open squares) or presence of 1.25 μ M Latrunculin A (closed diamonds). The percentage of cells in prometaphase and metaphase (PM + M) was determined at the times indicated ($n = 150$). (D) Representative images of *nmt1-atb2-gfp* cells in mitosis either in the absence (left) or presence (right) of 1.25 μ M Latrunculin A. Cells are either before (top) or after (bottom) sister chromatid separation. Microtubules were visualized by live cell imaging.

kinetochores were determined using OpenLab software (Improvision, Coventry, United Kingdom) and downloaded to Microsoft Excel for analysis.

RESULTS

The SOC Is Activated by Selective Disruption of Actin Cables

Latrunculin A and Latrunculin B are potent inhibitors of actin polymerization *in vivo* (Spector *et al.*, 1983; Ayscough *et al.*, 1997). There have been several seemingly contradictory reports of the effects of Latrunculin A and Latrunculin B on cell cycle progression in fission yeast. For example, we have previously shown that addition of 10 μ M Latrunculin B to synchronized cultures of wild-type fission yeast cells does not block spindle formation but causes cells to delay in mitosis with unseparated sister chromatids and misoriented spindles (Gachet *et al.*, 2001, 2004). We attributed this effect to activation of a SOC. Conversely, other researchers have shown that addition of 10 μ M Latrunculin A blocks the G2/M transition by preventing cell growth (Rupes *et al.*, 2001). To resolve this issue, we examined the effect of various concentrations of Latrunculin A on actin structures and mitotic progression in synchronous wild-type *plo1-GFP* cells. In the absence of any treatment, both actin cables and actin patches are clearly visible. In the presence of 1.25 μ M Latrunculin A, actin cables were absent although actin patches were still evident, particularly at the cell tips (Figure

1A). A similar pattern was observed when cells were treated with 10 μ M Latrunculin B, the concentration used in our previous studies (Figure 2A; Gachet *et al.*, 2001, 2004). In the presence of 12.5 μ M Latrunculin A, both actin cables and patches were absent (Figure 1A). Whereas addition of 12.5 μ M Latrunculin A blocked cell growth and the appearance of Plo1 kinase on spindle poles, addition of 1.25 μ M Latrunculin A did not block mitotic entry but delayed cells in mitosis with Plo1 associated to both spindle poles separated by ~ 2.0 – 2.5 μ m (Figure 1B). To examine more accurately the mechanism by which spindle misorientation regulates the timing of anaphase A, we constructed a strain that expresses a centromere-associated protein, Ndc80, tagged with GFP and a spindle pole body-associated protein, Cdc11, tagged with CFP (Krapp *et al.*, 2001; Wigge and Kilmartin, 2001). This strain enabled us to measure the period of time cells remained in prometaphase and metaphase (with kinetochores between but not adjacent to separated spindle poles). Addition of 1.25 μ M Latrunculin A did not delay separation of spindle poles, confirming that the G2/M transition is not affected under these conditions (Figure 1C). However, in the presence of 1.25 μ M Latrunculin A cells remain in prometaphase or metaphase for approximately twice as long than in control cells (as judged by the area under the curve) (Figure 1C). Under these conditions, astral microtubules undergo more erratic phases of growth and catastrophe

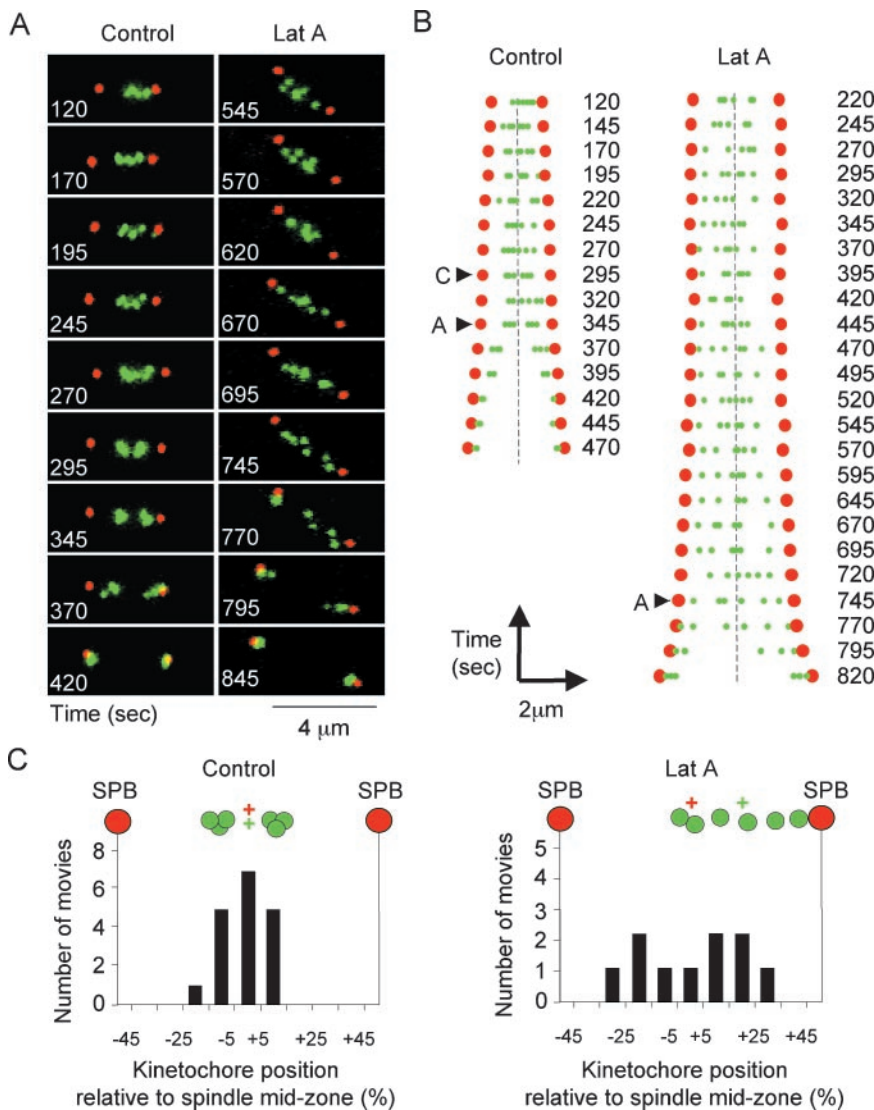


Figure 2. Latrunculin A prevents congression of centromeres to the spindle midzone. (A) Images from a movie of *ndc80-gfp cdc11-cfp* cells grown either in the absence (control) (Video 1) or presence of 1.25 μ M Latrunculin (Lat A) (Video 2). The localization of kinetochores (green) and spindle pole bodies (red) is shown at the times indicated. (B) Analysis of images from movies of *ndc80-gfp cdc11-cfp* cells grown either in the absence (control) (Video 1) or presence of 1.25 μ M latrunculin (Lat A) (Video 2). The localization of kinetochores (green) and spindle pole bodies (red) is shown at the times indicated. The position of each of the six kinetochores (green circles) relative to the positions of the two spindle pole bodies (red circles) is represented at 25-s intervals both for the untreated cell (control) and that treated with Lat A. The distance between each kinetochores and spindle pole bodies is plotted on the x-axis and time is shown in the y-axis. The spindle midzone (equi-distant between the poles) is shown as a dotted line. The point of chromosome congression (c) and anaphase onset (a) are shown. (C) Measurement of the central position of kinetochores (green cross) relative to the spindle midzone (red cross) taken 50 s before the onset of anaphase in movies of *ndc80-gfp cdc11-cfp* cells in the absence (control, left) or presence (Lat A, right) of 1.25 μ M Latrunculin A.

and are thus more difficult to visualize (Figure 1D). We find that, whereas 95% of spindles were aligned within 30° of the longitudinal axis at anaphase onset in control cells, only 51% of spindles were properly oriented in the presence of 1.25 μ M Latrunculin A. This confirms our previous observations that disruption of the actin cytoskeleton disrupts spindle rotation (Figure 1D, Gachet *et al.*, 2001; Gachet *et al.*, 2004). We conclude that whereas complete disruption of the actin cytoskeleton blocks cell growth, selective disruption of actin cables inhibits spindle rotation, presumably by inhibiting the formation of the cytokinetic actomyosin ring, and delays anaphase onset without effecting the G2/M transition. These data resolve the discrepancy between previous studies (Gachet *et al.*, 2001; Rupes *et al.*, 2001).

To circumvent the need to attain a critical cell size, Rajagopalan *et al.* (2004) analyzed the effect of 50 μ M Latrunculin A on cell cycle progression in *cdc25-22* cells and concluded that, under these conditions, Latrunculin A specifically activates the SOC. This prompted us to carry out a careful study of the effect of various concentrations of Latrunculin A on mitotic entry and progression in these cells. First, *cdc25-22* cells blocked at the G2/M transition were treated with a range of Latrunculin A concentrations and then stained with phalloidin. Whereas both

actin cables and actin patches were clearly visible in the absence of drug, addition of 2.5 μ M Latrunculin A disrupted actin cables, although actin patches were still visible (Supplementary Figure 1A). Addition of 5 μ M or higher Latrunculin A disrupted both actin cables and actin patches (Supplementary Figure 1A). To examine the effect of the various concentrations of Latrunculin A on mitotic entry and progression, *cdc25-22 plo1-GFP* cells were arrested in late G2 and released to the permissive temperature. Plo1 binds to spindle pole bodies (SPBs) only as cells enter mitosis and this requires activation of the Cdc2/Cdc13 kinase (Mulvihill *et al.*, 1999). Addition of 12.5 μ M Latrunculin A delayed chromosome separation (Supplementary Figure 1B) but did not affect association of Plo1 to spindle poles (Supplementary Figure 1C). However, at higher concentrations, Latrunculin A blocks the association of Plo1 to spindle poles and mitotic entry (Supplementary Figure 1, B and C). The IC_{50} value for this effect is 43 μ M, >10 times greater than the IC_{50} value of Latrunculin A for the disruption of actin structures in vivo. These data strongly suggest that at high concentrations, Latrunculin A inhibits mitotic entry by a mechanism that is unrelated to its effects as an inhibitor of actin polymerization.

Latrunculin Impedes Congression of Centromeres to the Spindle Midzone

To examine specifically the role of spindle mispositioning on mitotic progression, all further experiments were performed with 1.25 μ M Latrunculin A. We previously showed that separation of chromosome arms, as judged by examining the separation of a GFP marker integrated 30 kb from centromere 1 (*lys1::lacO his7::GFP-NLS-lacI*), is inhibited by addition of 10 μ M Latrunculin B (Gachet *et al.*, 2001). To investigate the effect of 1.25 μ M Latrunculin A on mitotic progression, we filmed kinetochore dynamics during mitosis in *ndc80-gfp cdc11-cfp* cells. Single cell analysis revealed that cells remained in prometaphase and metaphase for 11 ± 2 min, and this was extended to 20 ± 3 min in the presence of 1.25 μ M Latrunculin A (Figure 2). In the absence of drug, kinetochores make rapid oscillatory movements between the spindle poles during phase 2 (Figure 2A and Video 1). The position of each kinetochore was measured by fluorescence intensity and mapped relative to the position of the two spindle pole bodies for each frame of the movie (Figure 2B and Video 1), showing that the six kinetochores congress into two bunches of three either side of the spindle midzone ~ 50 s before the onset of anaphase A (Figure 2, A and B). The position of the kinetochores at congression was within the central 30% of the mitotic spindle in 17 of 18 cells filmed (Figure 2C). When anaphase takes place, each set of three sister chromatids arrive at their respective spindle pole bodies simultaneously. In the presence of Latrunculin A, kinetochores continue to oscillate between the spindle poles, suggesting that kinetochores are still under tension and no congression to the spindle midzone was observed before anaphase onset (Figure 2, A and B, and Video 2). In addition the spindle was longer (3.5 μ m) when anaphase took place. In 10 independent movies, kinetochores were found to separate at more diverse positions on the mitotic spindle (Figure 2C). These results demonstrate that centromeres congress to the spindle midzone before anaphase onset, and this is disrupted when the actin cytoskeleton is disturbed.

Latrunculin Inhibits Degradation of Spindle-associated Cyclin B and Securin

In a previous study, we found that 10 μ M Latrunculin B delays the degradation of Rad21, a component of the cohesin complex, suggesting that activation of Separase (Cut1) is delayed (Gachet *et al.*, 2001). However, Latrunculin B did not inhibit the bulk degradation of either Cut2 or Cdc13 (Cyclin B), as judged by Western blots of cell extracts (Gachet *et al.*, 2001). This led us to believe that Latrunculin B does not inhibit the action of APC. However, we could not discount the possibility that a small population of Cdc13 and Cut2 may remain undegraded during the delay or that Cdc13 and Cut2 relocate to an insoluble fraction during mitosis. To address this question we analyzed Cdc13 and Cut2 levels microscopically using synchronized *cdc13-CFP* and *cut2-GFP* strains (Tatebe *et al.*, 2001). In late G2 a strong signal of Cdc13 is observed in both chromatin and nucleolar domains, as observed previously (Alfa *et al.*, 1990; Tatebe *et al.*, 2001). As cells enter mitosis, the strong nucleolar staining diminishes, leaving a weaker nuclear signal (Figure 3, A and B). At the same time, Cdc13 concentrates at the unseparated spindle pole bodies. Cdc13 remains associated with chromatin and on the spindle and spindle poles until just before anaphase when it disappears, suggesting that Cdc13-GFP is effectively ubiquitinated by APC at this time (Alfa *et al.*, 1990; Tatebe *et al.*, 2001; Figure 3, A and B). In the presence of 1.25 μ M Latrunculin A, early relocalization and degrada-

tion of Cdc13 was not affected, although a proportion of Cdc13 remained associated to the spindle and spindle pole bodies for a longer period than in the control (Figure 3, A and B). Quantitative analysis indicates that $<20\%$ of the total Cdc13-GFP observed in G2 cells remains on the spindle and spindle pole bodies during metaphase. In movies of single cells, we also observed that Cdc13 remains associated to the spindle for longer in the presence of Latrunculin A (18 min) than in the control (8 min) (Supplementary Figure 2). Cut2 is located in the nucleus of G2 cells and associates only transiently to the spindle in M phase, as observed previously (Funabiki *et al.*, 1996a; Tatebe *et al.*, 2001; Figure 3, C and D). Addition of Latrunculin A resulted in an extended metaphase delay with Cut2 associated to the mitotic spindle (Figure 3, C and D). Cut9 is a core component of the APC in fission yeast (Funabiki *et al.*, 1996b). We find that Cdc13 and Cut2 associate strongly to mitotic spindles in *cut9-366* cells at the restrictive temperature, suggesting that this is an important site of APC action (our unpublished data). These results suggest that Latrunculin A inhibits sister chromatid separation by delaying activation of the APC.

Latrunculin Activates a Bub1-, Bub3-, and Mad3-dependent Anaphase Checkpoint

We previously showed that addition of 10 μ M Latrunculin B delays nuclear separation in cells lacking the Mad2 spindle assembly checkpoint protein (Gachet *et al.*, 2001). Because Latrunculin A delays the degradation of spindle-associated Cdc13 and Cut2, we undertook a careful investigation of the role of SAC proteins in delaying anaphase under these conditions. Components of the SAC include Mad1, Mad2, Mad3, Bub1, Bub3, and Mph1 (the homologue of budding yeast Mps1) (He *et al.*, 1997, 1998; Bernard *et al.*, 1998; Ikui *et al.*, 2002; Millband and Hardwick, 2002). Each of these genes was individually deleted in a *ndc80-gfp cdc11-cfp* strain to visualize centromere and SPB dynamics and, by this means, to assess the time spent in prometaphase and metaphase. In agreement with previous observations, Latrunculin A delayed the onset of anaphase in cells lacking Mad2 to a similar extent to that observed in control cells (Figure 4A). By contrast, addition of 1.25 μ M Latrunculin A failed to impose a metaphase delay in cells lacking Bub1 (Figure 4B). To confirm this result, we filmed individual *mad2 Δ ndc80-GFP cdc11-CFP* and *bub1 Δ ndc80-GFP cdc11-CFP* cells in mitosis. In the absence of Mad2 metaphase lasted ~ 6 min, and this was extended to 16 min in the presence of Latrunculin A (Supplementary Figure 3A). By contrast, in the absence of Bub1, metaphase lasted 4 min, and this was not extended in the presence of Latrunculin A (Supplementary Figure 3B). To examine whether the failure of *bub1 Δ* cells to delay in metaphase was due to the role of Bub1 in APC inactivation, Cdc13-GFP localization was monitored through mitosis. Whereas addition of Latrunculin A caused Cdc13 to persist on mitotic spindles in the absence of Mad2 (Figure 4C), it failed to do so in the absence of Bub1 (Figure 4D). This suggests Bub1 acts independently of Mad2 to block activation of the APC.

We next examined the length of prometaphase and metaphase in *bub1(K762M) ndc80-gfp cdc11-cfp* cells, which express an inactive Bub1 kinase. We found that Latrunculin A was unable to delay sister chromatid separation in these cells, suggesting that the catalytic activity of Bub1 kinase is required to delay anaphase under these conditions (Figure 5A). Furthermore, no anaphase delay was observed by addition of Latrunculin A to cells lacking either Bub3 (Figure 5B) or Mad3 (Figure 5C). However, addition of Latrunculin A to cells lacking Mad1 delayed the onset of anaphase as in wild-type cells (Figure 5D). We also observed a delay in cells lacking Mph1, but this was reproducibly less pronounced than in wild-type cells or in cells

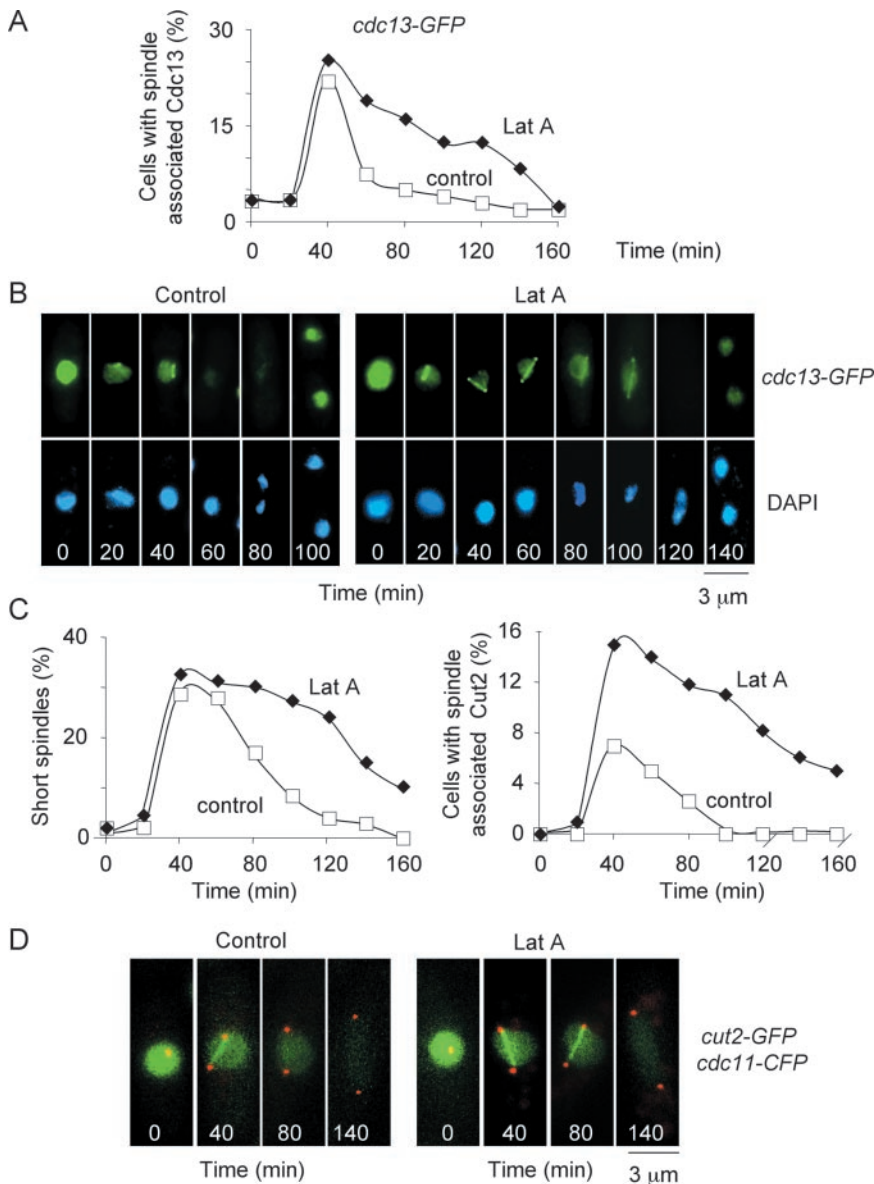


Figure 3. Latrunculin A delays the destruction of spindle associated Cdc13 (Cyclin B) and Cut2 (Securin). (A) Synchronized *cdc13-gfp* cells were incubated in the absence (open squares) or the presence of 1.25 μ M Latrunculin A (closed diamonds). The percentage of cells displaying spindle associated Cdc13 was determined ($n = 150$). (B) Representative images of *cdc13-gfp* cells from A showing the localization of Cdc13 at the times indicated in the absence or presence of 1.25 μ M Latrunculin A. (C) Synchronized *cut2-gfp cdc11-cfp* cells were incubated either in the absence (open squares) or the presence (closed diamonds) of 1.25 μ M Latrunculin A. The percentage of cells with either separated SPBs (left) or spindle associated Cut2 (right) was determined at the times indicated ($n = 150$). (D) Representative images of *cut2-gfp cdc11-cfp* cells from (C) showing the localization of Cut2 (green) or spindle pole bodies (red) at the times indicated, either in the absence or presence of 1.25 μ M Latrunculin A.

lacking Mad1 or Mad2 (Figure 5E). Note that in these experiments addition of 1.25 μ M Latrunculin A completely blocks the appearance of septa (Figure 5, A–E). These results suggest that disruption of the actin cytoskeleton delays activation of the APC via the Bub1, Bub3, and Mad3 spindle assembly checkpoint proteins.

Latrunculin Prolongs the Association of Bub1 with Kinetochores

The differential requirement for Mad2 and Bub1 in monitoring spindle mispositioning prompted us to examine their cellular location relative to centromeres. To do this, we constructed *mad2-GFP ndc80-CFP* and *bub1-GFP ndc80-CFP* strains. In control cells, Mad2 is observed throughout the nucleus in G2 and then, as cells enter mitosis, relocates to a region underlying the unseparated spindle pole that colocalizes with kinetochores, and it remains in this location as the spindle forms. Mad2, however, does not colocalize with kinetochores during metaphase (Figure 6A). By contrast, Bub1 remains associated with all kinetochores in promet-

aphase and metaphase, and the Bub1-GFP signal only diminishes a few minutes before anaphase onset (Figure 6B). To confirm these observations, we constructed *mad2-GFP cdc11-CFP* and *bub1-GFP cdc11-CFP* strains and examined the localization of Mad2 and Bub1 relative to the position of spindle poles. We found that Mad2 colocalizes with centromeres only in early mitosis and then is seen most prominently with only one of the separated SPBs (Supplementary Figure 4). Bub1, however, is broadly nuclear in G2, localizes to a region underlying the unseparated SPB in early mitosis, and then is found between the two SPBs, which are separated by a <2.5 - μ m spindle until it disappears shortly before anaphase (Supplementary Figure 4). Mad2 localization was monitored in *mad2-gfp cdc11-cfp* cells during an unperturbed mitosis or in the presence of 1.25 μ M Latrunculin A or a sub-lethal dose of the microtubule-depolymerizing drug benomyl. Although Mad2 remained bound to both the SPB and kinetochores for longer in the presence of benomyl, the localization of Mad2 was unaffected by addition of Latrunculin A (Figure 6C). By contrast, treatment of synchronous

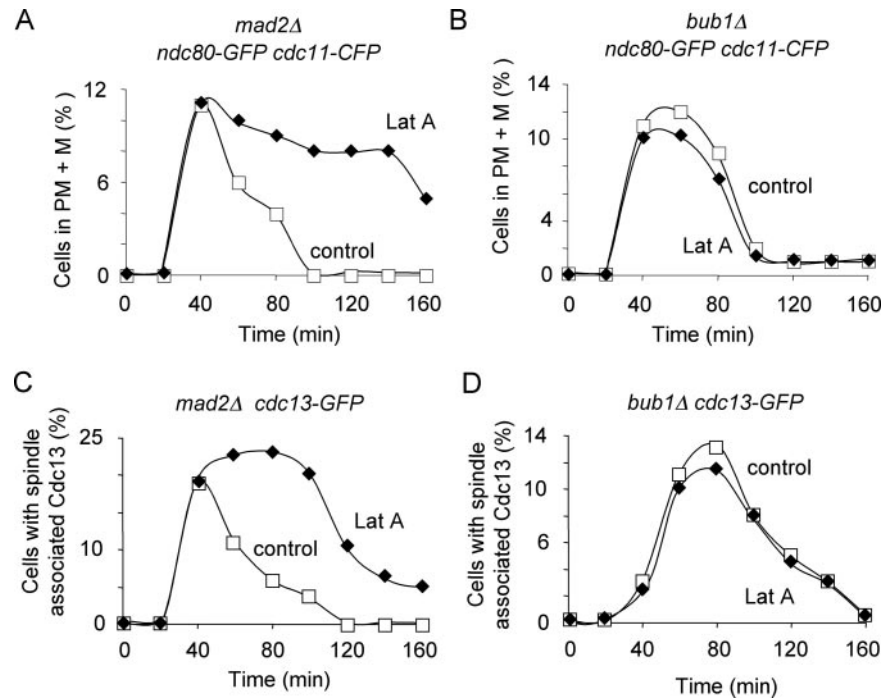


Figure 4. Latrunculin A inhibits APC and anaphase onset via a Bub1-dependent but Mad2-independent mechanism. (A) Synchronized *mad2Δ ndc80-gfp cdc11-cfp* and (B) *bub1Δ ndc80-gfp cdc11-cfp* cells were incubated either in the absence (open squares) or the presence (closed diamonds) of 1.25 μ M Latrunculin A. The percentage of cells in prometaphase and metaphase (PM + M) was determined at the times indicated ($n = 150$). Synchronized *mad2Δ cdc13-gfp* (C) or *bub1Δ cdc13-gfp* (D) cells were incubated either in the absence (open squares) or the presence (closed diamonds) of 1.25 μ M Latrunculin A. The percentage of cells showing spindle associated Cdc13 was determined at the times indicated ($n = 150$). Similar results were obtained from six independent experiments.

populations of *bub1-gfp ndc80-cfp* cells with 1.25 μ M Latrunculin A caused Bub1 to remain associated with kinetochores for longer than in control cells (Figure 6D). This was confirmed by live imaging of individual *bub1-gfp cdc11-cfp* cells (Figure 6E). Whereas we observed a maximum of two clusters of Bub1 dots in control cells, we frequently observed cells with up to 6 Bub1 dots for an extended period in the presence of Latrunculin A (Figure 6E). These data suggest that the Bub1 kinase is required at kinetochores to prevent anaphase onset when the actin cytoskeleton is disturbed.

The SOC is activated by disruption of astral microtubule contact with the cortical actin cytoskeleton

In previous studies, we showed that disruption of astral microtubule interaction with the cell cortex delays the timing of chromosome separation (Gachet *et al.*, 2001, 2004). To examine the delay in anaphase A onset more accurately, we monitored centromere dynamics in the actin mutant *cps8-188* and in *cdc11-123* cells, in which astral microtubule attachment to the spindle pole and spindle rotation are compromised (Ishiguro and Kobayashi, 1996; Krapp *et al.*, 2001; Gachet *et al.*, 2004). Single cell analysis revealed that *cps8-118 ndc80-gfp* cells remained in prometaphase and metaphase for 20 ± 3 min (Figure 7A), longer than in wild-type cells at the same temperature, but similar to that observed in wild-type cells in the presence of 1.25 μ M latrunculin A (Figure 2), consistent with our previous observations (Gachet *et al.*, 2004). By contrast, the length of prometaphase and metaphase was 8 ± 2 min in *bub1Δ cps8-118 ndc80-gfp* cells (Figure 7A) but unchanged in *mad2Δ cps8-118 ndc80-gfp* cells (our unpublished data). Similarly, the length of prometaphase and metaphase in *cdc11-123* cells was longer than in wild-type cells at the same temperature, and this was abolished by inactivation of Bub1 (Figure 7B; Gachet *et al.*, 2004). We find that Bub1 only binds kinetochores for ~ 4 min in wild-type cells. For this reason, Bub1 is bound to kinetochores in a small percentage of cells even in highly synchronous populations (Figure 7C). Nevertheless, we find that

Bub1 colocalizes kinetochores for longer in *cps8-188* and *cdc11-123* cells than in control cells at the same temperature (Figure 7C). These data strongly argue that Bub1 imposes an anaphase delay at kinetochores when astral microtubule contact with the cell cortex, and thus spindle position, is disturbed.

DISCUSSION

We previously proposed that addition of Latrunculin B activates a SOC in fission yeast that dictates the timing of chromosome separation (Gachet *et al.*, 2001). In agreement with this, we have found that astral microtubules interact with the cytokinetic actomyosin ring and that disruption of this process both prevents spindle alignment and delays anaphase onset (Gachet *et al.*, 2004). In this article, we confirm by live imaging of *ndc80-gfp cdc11-cfp* cells that addition of Latrunculin A, at concentrations that specifically disrupt actin cables, delays the separation of sister chromatids. This effect is also observed *cps8-188* cells, in which the cortical actin cytoskeleton is disturbed, and in *cdc11-123* cells, in which nucleation of astral microtubules is partially disrupted (Gachet *et al.*, 2004). These results provide further evidence that the onset of anaphase in fission yeast is regulated by a checkpoint that is activated when interaction of astral microtubules with the cortical actin cytoskeleton is disturbed. In this article, we have examined in more detail the mechanism by which anaphase is delayed under these conditions.

Chromosomes are held together in interphase and early mitosis by a complex that includes the Rad21(Scc1) protein (Uhlmann *et al.*, 1999; Tomonaga *et al.*, 2000). We previously showed that Latrunculin B delays the degradation of Rad21 but does not inhibit the degradation of either Cut2 or Cdc13 (Cyclin B), as judged by Western blot of cell extracts (Gachet *et al.*, 2001). This led us to believe that Latrunculin B blocks sister chromatid separation without inhibiting the activation of APC. We reexamined this conclusion by visually exam-

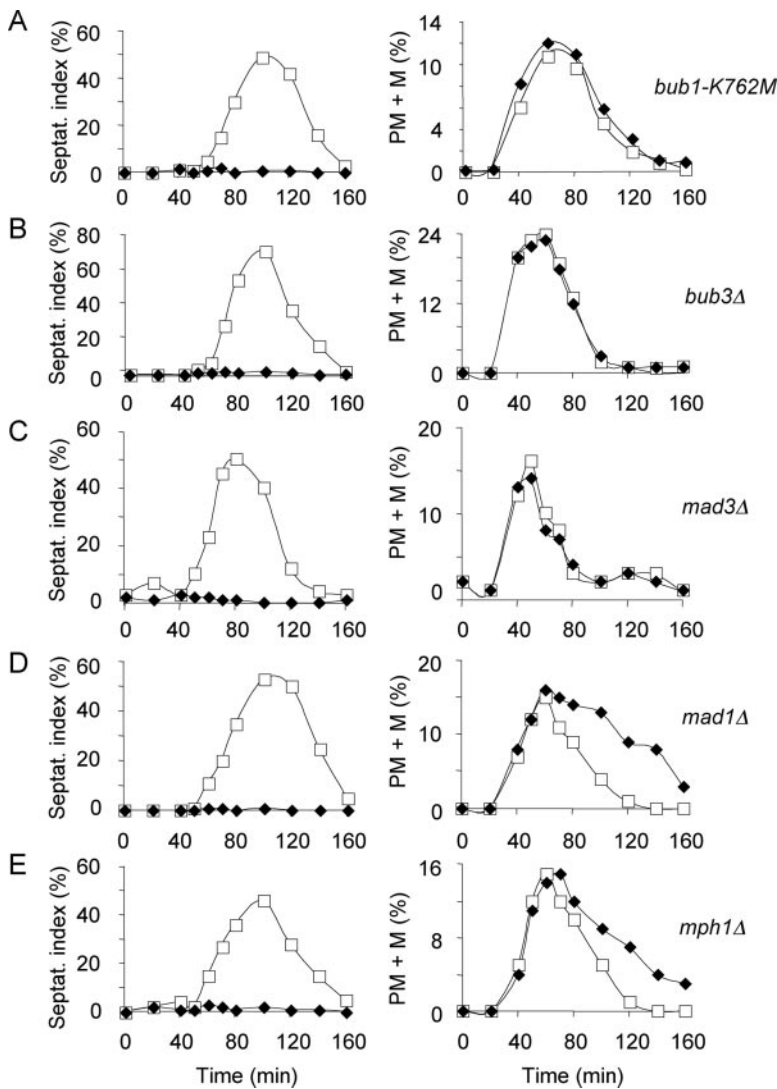


Figure 5. Anaphase delay induced by Latrunculin A requires Bub3 and Mad3 and the catalytic activity of Bub1. The following strains were synchronized in early G2 and transferred to fresh medium either in the absence (open squares) or the presence (closed diamonds) of 1.25 μM Latrunculin A. (A) *bub1-K762M ndc80-gfp cdc11-cfp*. (B) *bub3Δ ndc80-gfp cdc11-cfp*. (C) *mad3Δ ndc80-gfp cdc11-cfp*. (D) *mad1Δ ndc80-gfp cdc11-cfp*. (E) *mph1Δ ndc80-gfp cdc11-cfp* cells. The percentage of cells with septa (left) or in prometaphase and metaphase (PM + M) (right) was determined at the times indicated (n = 150). Similar results were obtained from at least five independent experiments.

ining Cdc13 and Cut2 levels in *cdc13-gfp* and *cut2-gfp* cells treated with Latrunculin. Although we observe a substantial reduction (>80%) in Cdc13-GFP fluorescence in early mitosis, a proportion of Cdc13 remains strongly associated with short misoriented spindles and spindle pole bodies when cells enter mitosis. Importantly, spindle-associated Cdc13 disappears just before sister chromatid separation, suggesting that it is effectively ubiquitinated by APC at this time. It is conceivable that a proportion of Cdc13 is degraded by APC in early mitosis, as is Cyclin A in mammalian cells (Geley *et al.*, 2001). However, we note that Cdc13 degradation is observed in temperature-sensitive mutants of the APC, raising the possibility that a distinct degradation pathway acts on Cdc13 in early M phase (Chang *et al.*, 2001). Regardless, we also find that Cut2 is stabilized on mitotic spindles in the presence of Latrunculin, and this also disappears before anaphase onset. These results indicate that, in contrast to our previous conclusion (Gachet *et al.*, 2001), the APC is inhibited when astral microtubule contact with the medial actin cytoskeleton is disturbed.

It is well known that sister chromatid separation is inhibited by addition of microtubule-disrupting agents to cells. This causes the recruitment of Mad1, Mad2, Mad3, Mph1, Bub1, and Bub3 proteins to unattached kinetochores and

inhibition of APC (Yu, 2002; Zhou *et al.*, 2002; Cleveland *et al.*, 2003). Activation of the spindle assembly checkpoint and recruitment of Mad2 to kinetochores is also observed in mutants, such as *alp7/mia1*, in which the stability of both astral and spindle microtubules is perturbed. In these cells, Mad2 is required to maintain cell viability (Oliferenko and Balasubramanian, 2002; Sato *et al.*, 2003). By contrast, we found that in response to Latrunculin A, both the separation of sister chromatids and the degradation of spindle associated Cdc13 and Cut2 are delayed in cells lacking Mad1 or Mad2, but not in cells lacking Mad3 or Bub3. In accordance with this, we find that Bub1 colocalizes with kinetochores when spindles are misoriented but Mad2 does not. Instead, Mad2 localizes to a region underlying the unduplicated spindle pole body in early mitosis and colocalizes with only one pole during spindle elongation, as observed by others (Garcia *et al.*, 2002). It remains to be determined whether this is because Mad2 remains closely associated only to the old spindle pole. Importantly, anaphase onset and the disappearance of Bub1 from kinetochores is delayed in *cps8-188* and *cdc11-123* cells and the anaphase delay in these mutants is dependent on Bub1 but not Mad2. These results suggest that spindle assembly and spindle orientation are monitored by distinct checkpoints that inhibit APC by an overlapping

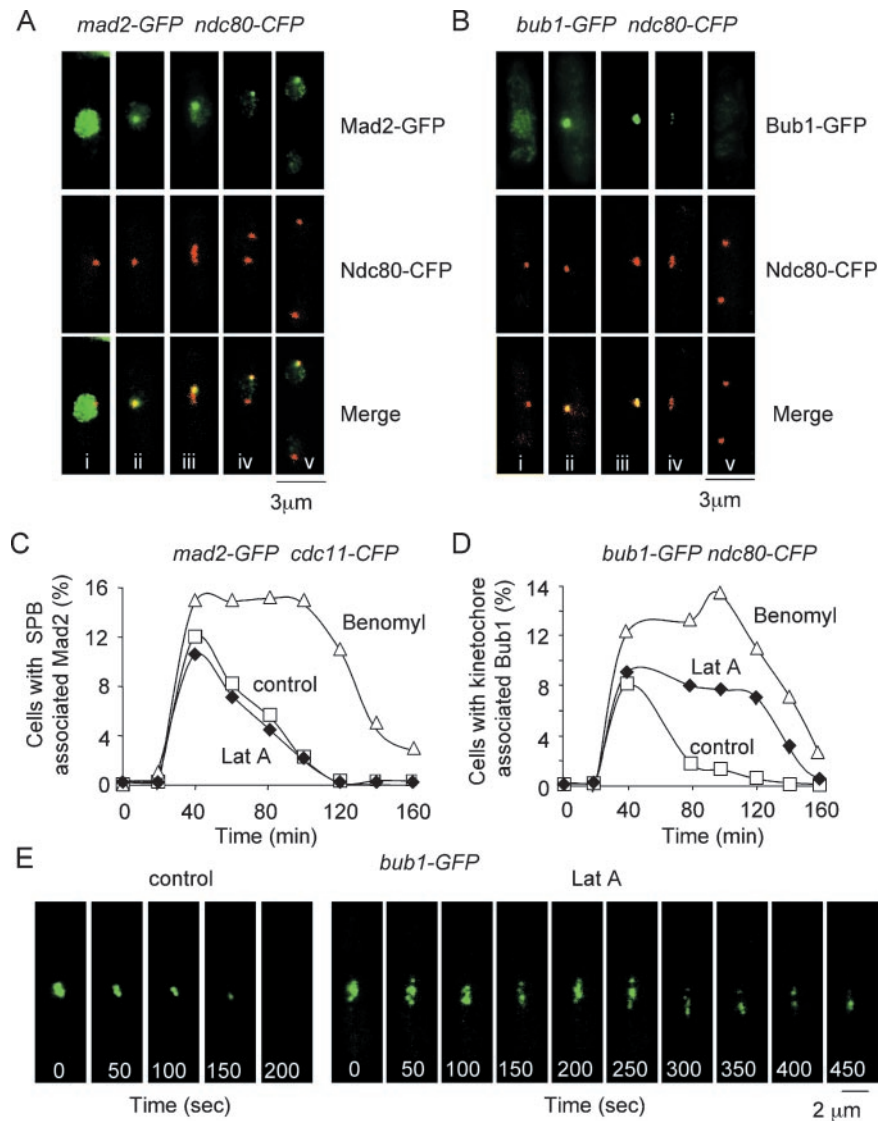


Figure 6. Bub1 associates to kinetochores when the SOC is activated. (A) Images of log phase cultures of *mad2-gfp ndc80-cfp* cells in either G2 (i), prometaphase (ii), metaphase (iii), anaphase (iv), or telophase (v) showing the localization of Mad2 (green) and Ndc80 (red). Colocalization is seen in yellow. (B) Images of log phase cultures of *bub1-gfp ndc80-cfp* cells in either G2 (i), various stages between prometaphase and metaphase (ii–iv), and telophase (v) showing the localization of either Bub1 (green) and Ndc80 (red). Colocalization is seen in yellow. (C) Synchronized *mad2-gfp cdc11-cfp* were transferred to fresh medium either in the absence (open squares) or the presence (closed diamonds) of 1.25 μ M Latrunculin A or with 3 μ M benomyl (open triangles). The percentage of cells displaying SPB associated Mad2 was determined at the times indicated ($n = 150$). (D) Synchronized *bub1-gfp ndc80-cfp* were transferred to fresh medium either in the absence (open squares) or the presence (closed diamonds) of 1.25 μ M Latrunculin A or with 3 μ M benomyl (open triangles). The percentage of cells displaying kinetochore associated Bub1 was determined at the times indicated ($n = 150$). (E) Images from movie of *bub1-gfp cdc11-cfp* cells incubated either in the absence (control) or the presence (Lat A) of 1.25 μ M Latrunculin A. Time zero is when the spindle became 2 μ m in length. Localization of Bub1 at various times is shown (green).

but nonidentical mechanism (Figure 8). We note that in budding yeast Bub1 and Bub3 bind kinetochores every cell cycle, whereas Mad1 and Mad2 only associate to kinetochores when spindles are damaged, indicating that components of the SAC are nonequivalent (Gillett *et al.*, 2004). Intriguingly, a complex containing Cdc20, BubR1(Mad3), and Bub3, but lacking Mad2, has been isolated from tissue culture cells as a potent inhibitor of APC activity in vitro (Tang *et al.*, 2001). We are currently generating reagents to examine whether a similar complex inhibits APC in fission yeast when spindles are mispositioned.

Recently, Rajagopalan and colleagues have suggested, by analysis of a mutant in the kentrin-like spindle pole component Pcp1 [*pcp1*($\Delta 400$ –900)], that metaphase spindle position is monitored by the Bub1 but not Mad1 or Mad2 spindle assembly checkpoint proteins (Rajagopalan *et al.*, 2004). However, we find that, by analyzing kinetochore dynamics, the delay imposed by disrupting astral microtubule contact with the cell cortex is dependent not only on Bub1 but also on the Bub3 and Mad3 spindle assembly checkpoint proteins. Furthermore, whereas Rajagopalan and colleagues find that disruption of *mph1* abolishes the anaphase delay in *pcp1*($\Delta 400$ –900) cells, we find that addition of Latrunculin A

imposes an anaphase delay in cells lacking Mph1, although this is reproducibly less pronounced than in wild-type cells. One possibility is that the metaphase delay in *pcp1*($\Delta 400$ –900) cells is due to a mitotic defect other than spindle misorientation, because spindle alignment is apparently not affected in this mutant (Rajagopalan *et al.*, 2004). Alternatively, because mitotic progression in *pcp1*($\Delta 400$ –900) cells was judged only by staining of nuclei and spindles, the timing of sister chromatid separation may have been masked by an additional delay over anaphase B (spindle elongation). We suggest that a careful analysis of kinetochore dynamics in *pcp1*($\Delta 400$ –900) cells, such as that performed in this study, may help to resolve this issue. We point out that first, both Mad3 and Bub3 are necessary for Mps1(Mph1) to impose a metaphase arrest in budding yeast (Hardwick *et al.*, 1996), and second, Bub3 is required both for the association of Bub1 to kinetochores and for the checkpoint function of Bub1 (Farr and Hoyt, 1988; Taylor *et al.*, 1988; Warren *et al.*, 2002; Gillett *et al.*, 2004). In this regard, the localization of Bub1 in metaphase arrested *pcp1*($\Delta 400$ –900) cells will be of interest. In other experiments, we have found that addition of 50 μ M Latrunculin A to *cdc25-22* cells inhibits mitotic onset by a mechanism that is independent of

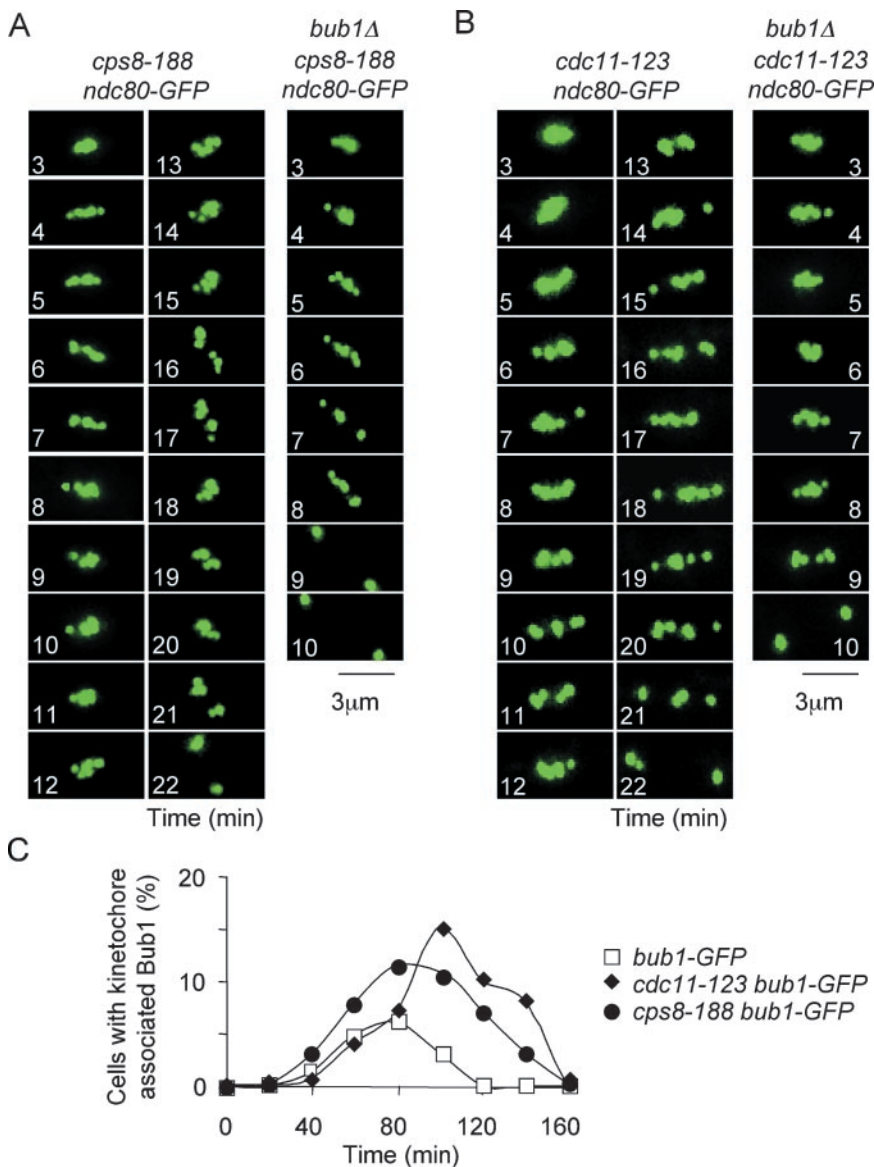


Figure 7. Bub1 monitors astral microtubule contact with the cell cortex. (A) Images from a movie of *cps8-188 ndc80-gfp* cells or *bub1Δ cps8-188 ndc80-gfp* cells in mitosis. The localization of kinetochores (green) is shown at the times indicated. Time zero is the beginning of phase 1 when kinetochores are first seen to separate. (B) Images from a movie of *cdc11-123 ndc80-gfp* cells or *bub1Δ cdc11-123 ndc80-gfp* cells in mitosis. The localization of kinetochores (green) is shown at the times indicated. Time zero is the beginning of phase 1 when kinetochores are first seen to separate. (C) Synchronized *bub1-gfp* (open squares), *cps8-188 bub1-gfp* (closed diamonds) or *cdc11-123 bub1-gfp* (closed circles) cells were incubated in fresh medium and the percentage of cells displaying Bub1 at kinetochores was then determined at the times indicated ($n = 150$). Similar results were obtained from three independent experiments.

its effects as an inhibitor of actin polymerization (Supplementary Figure 1). Thus, the delay in anaphase onset observed by Rajagopalan and colleagues may be due, at least in part, to a delay in mitotic entry. This may also explain why these researchers failed to identify a role for Mad3 and Bub3 in their experiments (Rajagopalan *et al.*, 2004).

In this and previous articles, we have shown that anaphase is delayed when the medial actin cytoskeleton is disturbed (Gachet *et al.*, 2001, 2004). We attributed this effect to activation of an SOC because spindle rotation is blocked and also there is no known role for actin at the kinetochore. Despite this, it is still unclear whether the SOC monitors astral microtubule contact with the cell cortex, the integrity of the cortical actin cytoskeleton or spindle angle itself. Recently, Rajagopalan and colleagues proposed that spindle position is monitored by a tension checkpoint at the spindle pole (Rajagopalan *et al.*, 2004). However, we find that sister kinetochores continue to undergo dynamic movement along the mitotic spindle when spindles are mispositioned, suggesting that the spindle is still under tension. So what do the Bub1, Bub3, and Mad3 checkpoint proteins sense? Intriguingly,

we have found that astral microtubules often interact with the cytokinetic actomyosin ring shortly before the onset of anaphase, an event that is necessary to align the mitotic spindle perpendicularly to the axis of cell division (Gachet *et al.*, 2004). One attractive possibility is that the SOC monitors attachment of astral microtubules with the cytokinetic actomyosin ring. This would be analogous to the role of SAC components in monitoring attachment of spindle microtubules to the kinetochore. If this is the case, a signal must be transmitted from the cell cortex to the kinetochore, because Bub1 binds kinetochores in SOC-activated cells. Clearly, further experimentation will be needed to test this and other possibilities.

In mammalian cells, chromosomes align on a metaphase plate that is formed equidistant between the centrosomes and at the site of the future cleavage furrow (Rieder and Salmon, 1994). Similarly, in budding yeast, kinetochores congress to the spindle midzone as the spindle is positioned across the bud neck, suggesting that a pseudometaphase plate also exists in fungi (Pearson *et al.*, 2001). In agreement with this, we find that centromeres congress to the spindle

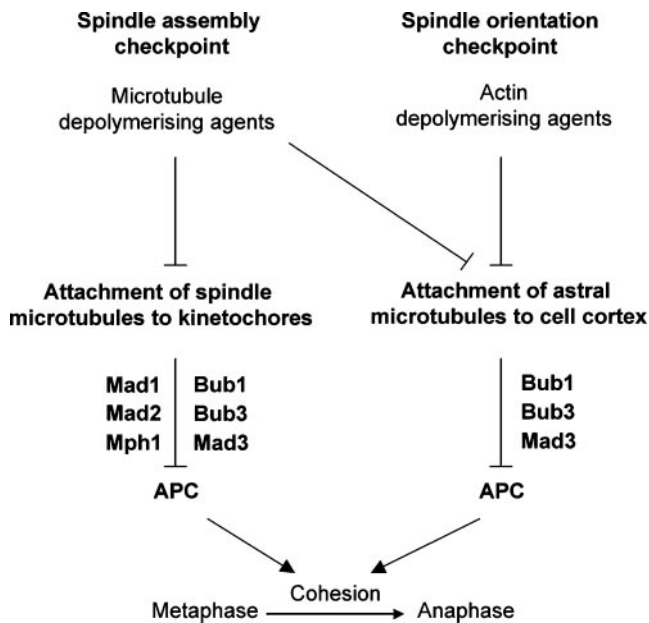


Figure 8. Spindle assembly and orientation checkpoints in fission yeast. The spindle assembly checkpoint inhibits the APC via the Mad1, Mad2, Mph1, Mad3, Bub1, and Bub3 proteins in response to a lack of bipolar attachment of kinetochores to spindle poles. The spindle orientation checkpoint inhibits APC by a pathway that requires Bub1, Bub3, and Mad3 but not Mad1 or Mad2. Microtubule depolymerizing agents activate both spindle assembly and spindle orientation checkpoints. Actin depolymerizing drugs only disrupt astral microtubule interaction with the cell cortex and activate only the spindle orientation checkpoint.

midzone just before sister chromatid separation in fission yeast. Because anaphase onset follows shortly after the establishment of a metaphase plate, it is thought that chromosome congression is monitored by a checkpoint that dictates the timing of anaphase. Notably, some SAC components such as Bub1, BubR1(Mad3), and Bub3 bind kinetochores at the metaphase plate, whereas Mad1 and Mad2 do not (Waters *et al.*, 1998; Campbell and Hardwick, 2003). We find that in the presence of Latrunculin A, or in mutants in which astral microtubule contact with the cell cortex is disturbed, chromosome congression does not take place. This may be a secondary consequence of an anaphase delay. Alternatively, a subset of spindle assembly checkpoints, including Bub1, Bub3, and Mad3, may ensure both that chromosomes congress to the spindle midzone (metaphase plate) and that the spindle is positioned perpendicularly to the cytokinetic actomyosin ring before anaphase onset. If this is the case, the spindle orientation checkpoint in fission yeast may be indistinguishable from the checkpoint that ensures formation of a metaphase plate in animal cells.

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